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# *WTH3*, a new member of the *Rab6* gene family, and multidrug resistance

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**Abstract**

The *WTH3* gene was obtained by a DNA fragment isolated by the methylation-sensitive representational difference analysis technique due to its hypermethylation in the human multidrug resistant (MDR) breast cancer cell line MCF7/AdrR. The *WTH3* gene product is 89% and 91% identical to the human Rab6 and Rab6c proteins, but possesses an elongated C-terminal region which contains 46 extra amino acids. Nevertheless, we consider the *WTH3* gene a new member of the *Rab6* gene family. Semi-quantitative reverse transcriptase–polymerase chain reaction results showed that *WTH3* was 15 and 4 times downregulated in MCF7/AdrR and MES-SA/Dx5, a human MDR uterine sarcoma cell line, as compared to their non-MDR parental cell lines. Permanent expression of the *WTH3* transgene in MDR cell lines increased to varying degrees their sensitivity to several anticancer drugs, which included doxorubicin, taxol, vinblastine, vincristine, and etoposide, as compared to the control sublines transfected with the empty vector. Flow cytometry and fluorescence microscope experiments suggest that the *WTH3* transgene stimulated the host's uptake and retention of DOX. Our results imply that the *WTH3* gene plays a role(s) in MDR phenotype development in vitro. 

*Keywords:* Methylation-sensitive representational difference analysis; G protein; Gene transfection; Drug resistance

## 1. Introduction

Although *MDR1* and *MRP* genes [1–6], as well as other gene products, such as lung resistance-related protein (LRP) and glutathione transferase [7–9], have been widely recognized as factors involved in MDR development, the clinical MDR phenomenon is still not completely understood. Clearly other unknown drug resistant mechanisms are at work [10,11]. Therefore searching for those mechanisms, with the help of modern techniques, may provide information of great importance in understanding the etiology of clinical MDR.

One strategy, which could be utilized to uncover these hidden systems, is the study of DNA methylation. This event suppresses gene expression which may cause distinct cellular phenotypes [12–14]. For example, a correlation between DNA methylation, gene expression, and drug resistance has been reported by several scientific groups [15–19]. In addi-

Abbreviations: aa, amino acid; bp, base pair(s); cDNA, DNA complementary to RNA; MS-RDA, methylation-sensitive representational difference analysis; MDR, multidrug resistance; MTT, 3-[4,5-dimethylthiazol-*Z*-yl]-2-5-diphenyl-tetrazolium bromide

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tion, a methylated site is usually found close to the 5′ end of the gene to be regulated [20–22], and therefore a differentially methylated DNA fragment could be a useful tool in the search for genes related to MDR. Considering this we employed methylation-sensitive representational difference analysis (MS-RDA) [23,24], which is an RDA [25] based technology, to search for DNA fragments which were hypermethylated in a MDR human breast cancer cell line, MCF7/AdrR, but not in its non-MDR parental cell line, MCF7/WT. Both cell lines were gifts from Dr. K. Cowan of the National Cancer Institute [26].

As a result, a hypermethylated DNA fragment, *W3*, was isolated by MS-RDA. The study of *W3* has led to the discovery of two genes, *Rab6c* [27] and *WTH3*. In this report we present our findings related to *WTH3*, which was acquired by performing the 3′-RACE (rapid amplification of cDNA ends) technique based on the *W3* sequence. *WTH3* is homologous to the human *Rab6* and *Rab6c*/*Rab6A′* genes [27–29] but possesses its own unique feature, an extended 46-amino-acid C-terminal region (recently, this gene has been documented by another group [30]). Consistent with its hypermethylated status, the *WTH3* gene was 15 times less expressed in MCF7/AdrR than in MCF7/WT cells. In addition, it was four times less expressed in the MES-SA/Dx5 cell line (its MDR phenotype was induced from the parental cell line, MES-SA, by doxorubicin (DOX) (ATCC Inc.) [31,32]) as compared to MES-SA. Research on the stable MCF7/AdrR and MES-SA/Dx5 cell lines which contained the *WTH3* transgene suggest that *WTH3* reversed the host cells' MDR phenotype to several anticancer drugs tested and increased DOX retention of the host cells. Our studies indicate that the *WTH3* gene, functioning as a negative regulator, was involved in the evolution of drug resistance in the working model systems. Whether *WTH3* is also involved in clinical drug resistance is currently under investigation.

## 2. Experimental procedures

### 2.1. Human cell lines and culture conditions

MCF7/AdrR, MCF7/WT, MES-SA/Dx5, and MES-SA cells were grown under the conditions as described [27].

### 2.2. MS-RDA

To study hypermethylation events in MCF7/AdrR cells, MCF7/WT DNA was used as tester, while MCF7/AdrR DNA was used as driver. MS-RDA was performed as described [23].

### 2.3. Amplicon and genomic methylation sensitive Southern analysis

Amplicon Southern blot was performed as described [23]. Two hundred and fifty ng of tester and driver amplicon DNA were utilized. Genomic methylation sensitive Southern blot was performed as described [23].

### 2.4. DNA Sequencing

Polymerase chain reaction (PCR) products were sequenced using a Dye-Labeled Sequence kit (Perkin Elmer) under conditions specified by the manufacturer.

### 2.5. Obtaining the WTH3 Gene by 3′-RACE

A unique sequence, 5′-GATGGAACAATCGGGCTTCG-3′ (PW3-1), which is located in the middle of the *W3* fragment, was designed as a PCR primer to obtain the 3′ end portion of the *WTH3* gene from a combined human cDNA library (Quick-Screen Human cDNA library Panel, Clontech). The antisense primer (PV-1), 5′-ACGACTCACTATAGGGCGAATTGGC-3′, was designed based on the vector sequence. PCR amplification was performed using the Advantage GC KlenTaqPolymerase Mix kit (Clontech) following the manufacturer's instructions. A 1.3 kb PCR product was generated and sequenced. Based on the sequencing information, another *W3*-specific primer, 5′-AAACAGTCAGCGAAGGGGGT-3′ (PW3-RACE1), which is close to the stop codon, was designed to perform RACE to obtain another PCR product to verify that the original PCR product is our true target. To acquire an intact gene, PW3-1 was paired with another primer, 5′-CCCAAGCTTGACTTTTTTTGTGCTTGTCAAGC-

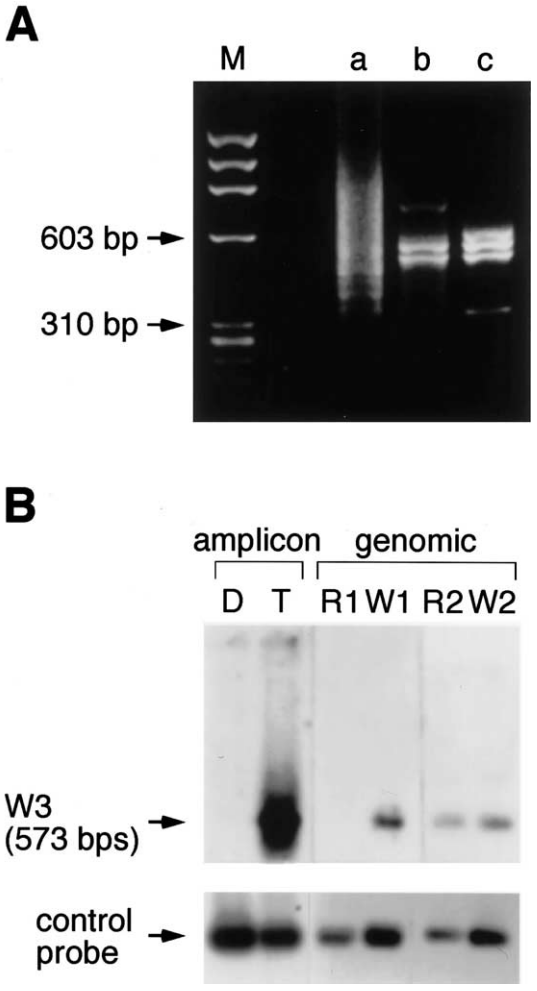


Fig. 1. (A) Agarose gel electrophoresis of amplicon and difference products generated by MS-RDA from MCF7/WT DNA. Lane M represents *Hae*III ϕ174 markers. Lane a represents the tester amplicon. Lanes b and c contain the difference products after first and second round subtraction hybridization/PCR amplification. (B) Results of the amplicon and genomic Southern blot for hypermethylated *W3*. The arrows indicate the fragments that are hybridized with the radiation labeled *W3* and control probes. In lanes D and T, each probe hybridizes with 250 ng of driver (MCF7/AdrR) and tester (MCF7/WT) amplicon DNA. In lanes R1 and W1, the *W3* probe hybridized with 6 μg of *Hpa*II-digested genomic DNA isolated from MCF7/AdrR and MCF7/WT cells, respectively. In Lanes R2 and W2, each probe hybridized with 6 μg of *Msp*I-digested genomic DNA also isolated from MCF7/AdrR and MCF7/WT. The completion of enzymatic digestion for all genomic DNAs was confirmed by a control probe which was a background DNA fragment isolated by MS-RDA.

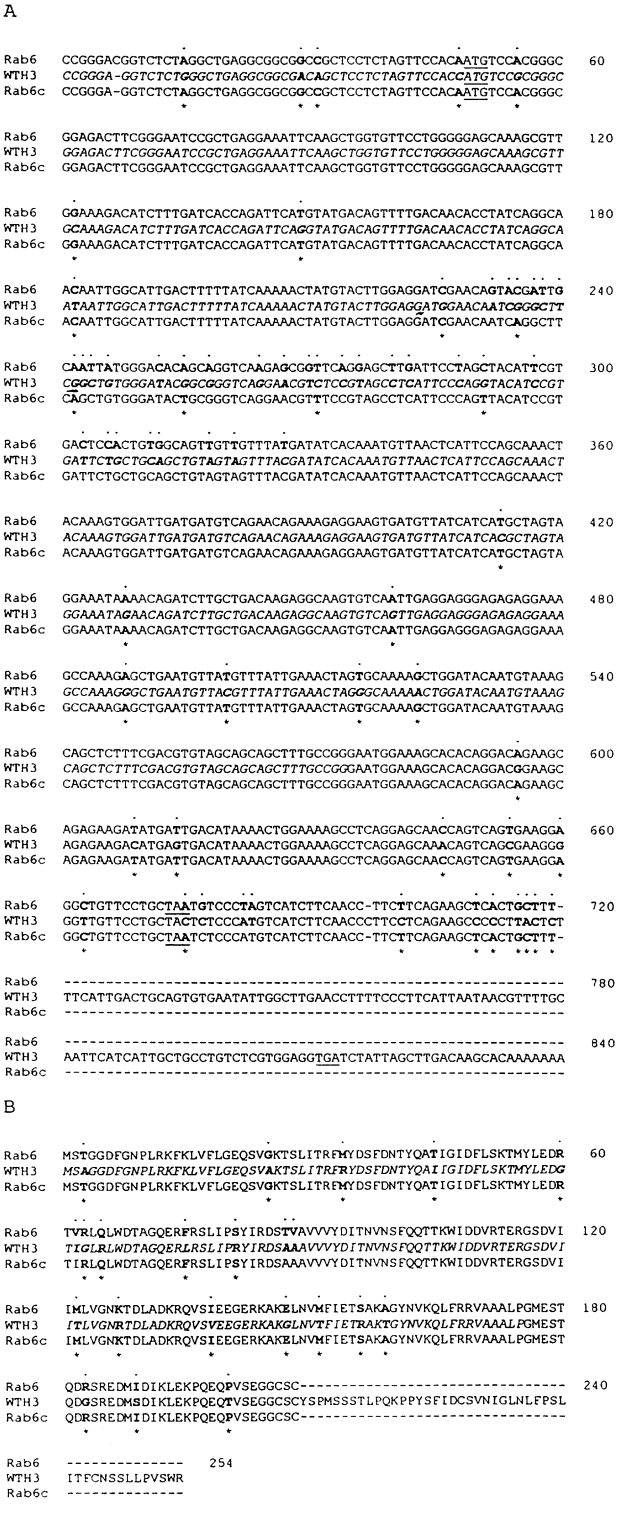

Fig. 2. The comparison of nucleotide sequences between *WTH3*, *Rab6*, and *Rab6c* (A) and the comparison of amino acid sequences between WTH3, Rab6, and Rab6c (B). The *W3* fragment isolated by MS-RDA is in italics. The nucleotide and amino acid substitutions between the three genes are in bold type. The differences between *WTH3* and *Rab6* and their products are marked with dots, while the differences between *WTH3* and *Rab6c* and their products are marked with stars. The start and stop codons of the genes are underlined.
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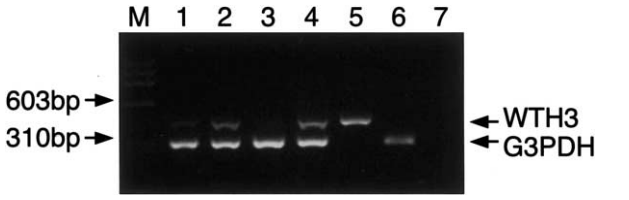


Fig. 3. Results of *WTH3*-specific SQRT–PCR. M represents *Hae*III ϕ174 markers. The cell lines used to prepare cDNA for simultaneously generated *WTH3* and *G3PDH* PCR products are indicated by numbers: 1, MES-SA/Dx5; 2, MES-SA; 3, MCF7/AdrR; 4, MCF7/WT. Lanes 5 and 6 contain *WTH3* and *G3PDH* PCR products generated from cDNA prepared from MCF7/WT cells, which served as positive controls. Lane 7 is the PCR negative control.

3′ (PW3-RACE2), which is located beyond the stop codon and followed by an artificial *Hin*dIII restriction enzyme site (for cloning purposes), to generate the 3′ end of the gene. This PCR product was digested by *Bgl*II, an endogenous site, and *Hin*dIII. To obtain the intact *WTH3* gene, the *Bgl*II/*Hin*dIII fragment was then subcloned into the pUC118-W3 plasmid containing the *W3* sequence, which was also digested by *Bgl*II, the corresponding endogenous site, and *Hin*dIII, which is in the polylinker, to release the truncated 3′ end region.

### 2.6. Semi-quantitative reverse transcriptase–polymerase chain reaction (SQRT–PCR) and RT–PCR

Total RNAs were isolated from the cell lines by the RNA isolation kit, RNA STAT-60 (TEL-TEST). The first-strand cDNA was synthesized by SuperScript Preamplification System Kit (Life Technologies). SQRT–PCR was performed by utilizing PW3-1, the *WTH3* gene sense primer, and the antisense primer, 5′-GCTGCTACACGTCGAAAGAGC-3′, while the cDNAs of MCF7/AdrR, MCF7/WT, MES-SA/Dx5, and MES-SA served as templates. The length of the *WTH3* PCR product was 341 bp. The sense and antisense primers for *G3PDH* (internal control) were 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. The length of the *G3PDH* PCR product was 284 bp. To confirm exogenous *WTH3* gene expression in the stable cell line, PW3-RACE1 was employed as the sense primer. A sequence in the polylinker of the pcDNA3.1 (Invitrogen) vector, 5′-CACTGTGCTGGATATCTGCAG-3′ (PV-2), was

Table 1
Effects of drugs on *WTH3* transfected MES-SA/Dx5 clone cells

| Drugs | ME-V | | | ME-2 | | | ME-4 | | | ME-7 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $IC_{50}$ (nM) (range, nM) | *N* | SF | $IC_{50}$ (nM) (range, nM) | *N* | SF (range, nM) | $IC_{50}$ (nM) | *N* | SF | $IC_{50}$ (nM) (range, nM) | *N* | SF |
| DOX | 770 (700–900) | 5 | 1 | 22 (9–26) | 5 | 35 | 93.3 (85–130) | 5 | 8.3 | 54 (45–100) | 5 | 14.3 |
| TAX | 615 (490–700) | 4 | 1 | 2.85 (1.92–3.0) | 4 | 216 | 28 (16–30) | 3 | 22 | 20.5 (9–25) | 3 | 30 |
| VBL | 59 (45–70) | 4 | 1 | 1.3 (0.3–1.5) | 7 | 45 | 7 (5–13) | 4 | 8.4 | 3.6 (2.8–5.8) | 4 | 16.4 |
| VCR | 408 (380–480) | 5 | 1 | 2.35 (0.2–3.2) | 11 | 174 | 30.7 (28–60) | 5 | 13 | 14.5 (6–35) | 5 | 28 |
| VP-16 | 8900 (6900–11000) | 4 | 1 | 340 (320–500) | 4 | 26 | 2300 (500–2700) | 4 | 4 | 1050 (600–1900) | 4 | 8.5 |
| CISP | 1400 (1200–1500) | 4 | 1 | 810 (640–840) | 4 | 1.7 | 1413 (1325–1775) | 4 | 1 | 1180 (420–1400) | 4 | 1.2 |

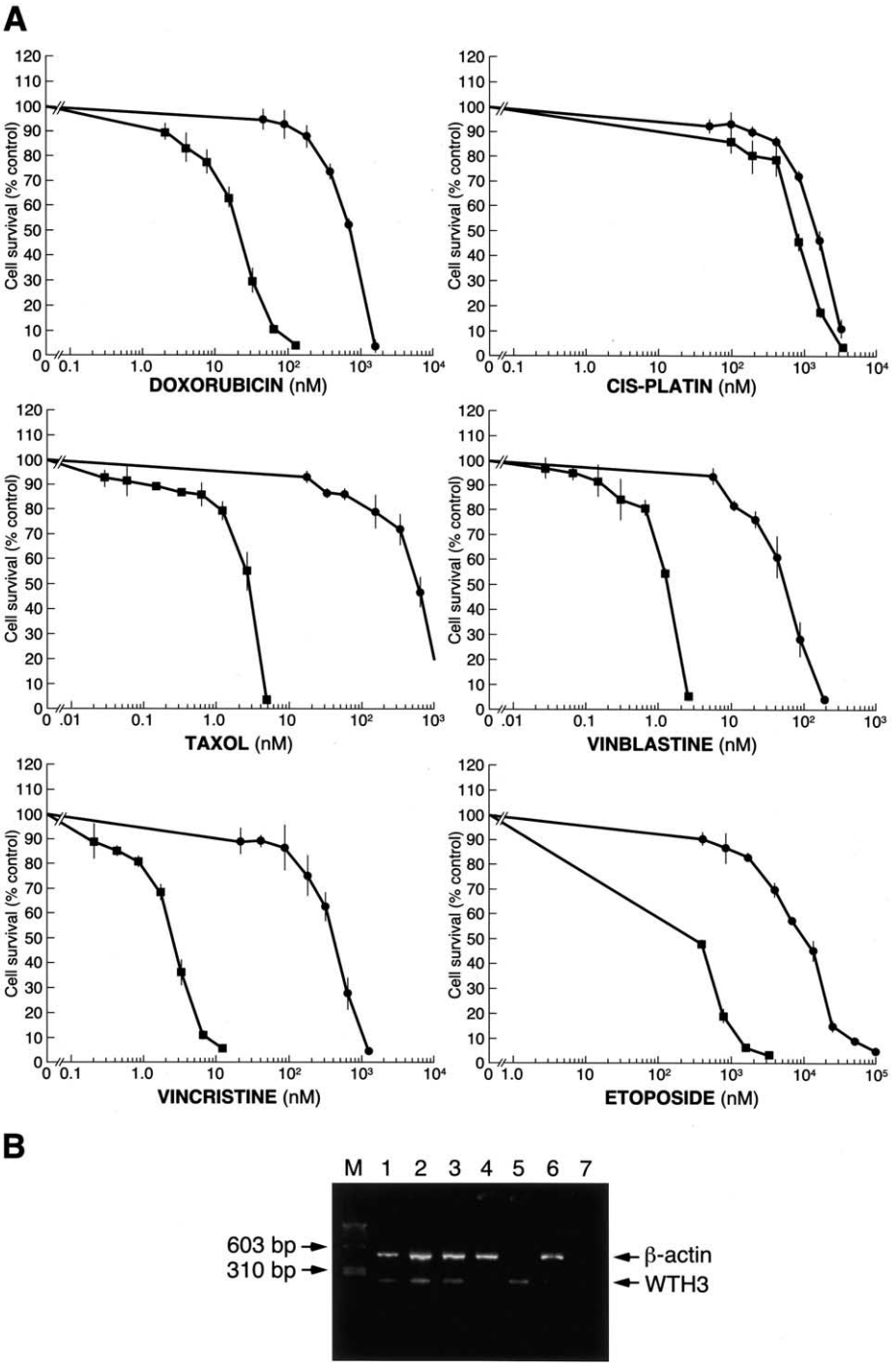


Fig. 4. Growth inhibition of ME-2 cells caused by different concentrations of DOX, CISP, TAX, VBL, VCR, and VP-16 (A) and QRT–PCR of the expressed exogenous *WTH3* gene in ME-2, ME-4, and ME-7 cells (B). (A) The line formed by linking the solid circles or squares represents the survival rate of ME-V and ME-2 cells (mean of more than four experiments), respectively. The thin bars represent standard deviation. (B) Lane M represents *Hae*III ϕ174 markers. Lanes 5 and 6 contain the positive controls for *WTH3* and *β-actin*. The product in lane 5 was generated by using cDNA prepared from ME-2 RNA and *WTH3*-specific primers. The product in lane 6 was generated from cDNA prepared from MES-SA/Dx5 cells. Lanes 1, 2, and 3 contain simultaneously generated *WTH3* and *β-actin* PCR products by utilizing cDNAs which were prepared from ME-2, ME-4, and ME-7 cells. Lane 4 only contains *β-actin* generated from cDNA prepared from ME-V cells, although *β-actin*- and *WTH3*-specific primers were simultaneously added. Lane 7 is the negative control.

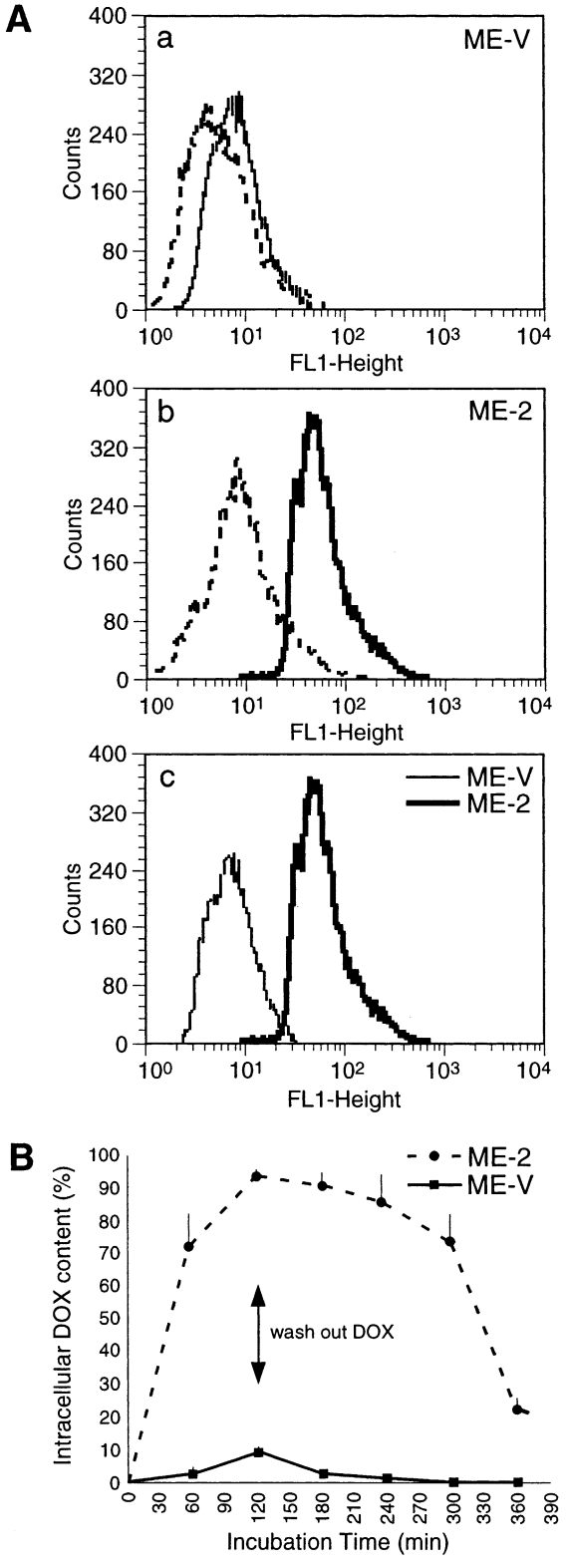


Fig. 5. Analysis of DOX uptake in ME-V and ME-2 cells by flow cytometry. DOX uptake in each cell group is presented by a peaked graph (A), and DOX uptake and retention in each group is presented by a curved graph (B). (A) Comparison of intercellular fluorescence in ME-V versus ME-2 cells. a, ME-V, at 0 (dashed line) and 2 h incubation with DOX (thin solid line); b, ME-2, at 0 (dashed line) and 2 h incubation with DOX (thick solid line); c, the comparison of accumulated DOX fluorescence in ME-V (thin solid line) and ME-2 (thick solid line) at 2 h incubation. (B) Cells were treated with DOX for 2 h and then washed. The cells remained in a DOX-free medium for up to 4 h. The intercellular fluorescence at different time points was measured by a flow cytometer. The solid and dashed lines represent ME-V and ME-2 cells, respectively. The solid points are the means of duplicated determinations. The vertical bars represent the upper or lower range at that data point.
←

used as antisense primer to synthesize the *WTH3* transgene fragment (226 bp). The sense and antisense primers for generating the *β-actin* fragment (495 bp) which served as a control were 5′-GACGACATG-GAAGATCTGG-3′ and 5′-ATCGGGCAGCTCG-TAGCTCTTCC-3′. The PCR and quantification of PCR products were performed as described [27].

### 2.7. Generation of stable cell lines

The PCR-generated *WTH3* gene was subcloned into the mammalian expression vector, pcDNA3.1, to create pcDNA3.1/WTH3. The gene sequence was confirmed by DNA sequencing. MCF7/AdrR and MES-SA/Dx5 cells grown to 60% confluence in 60-mm dishes were transfected with 2 μg of pcDNA3.1/WTH3, or pcDNA3.1 (control) plasmid using a calcium phosphate precipitation kit (5 Prime 3 Prime). The transfected MCF7/AdrR and MES-SA/Dx5 cells were maintained in medium supplied with 200 and 250 μg/ml of neomycin analogue G418 (Gibco), respectively. Stable G418 resistant populations were acquired after 2 weeks of selection. The individual clones were obtained by limiting dilution.

### 2.8. Cell growth inhibition

Approximately $1 \times 10^3$/well of *WTH3* or the empty vector transfectants were seeded in a 96-well plate (Corning Costar) and grown overnight. DOX, cisplatin (CISP), taxol (TAX), vinblastine (VBL), vincristine (VCR), and etoposide (VP-16) (Sigma) were se-

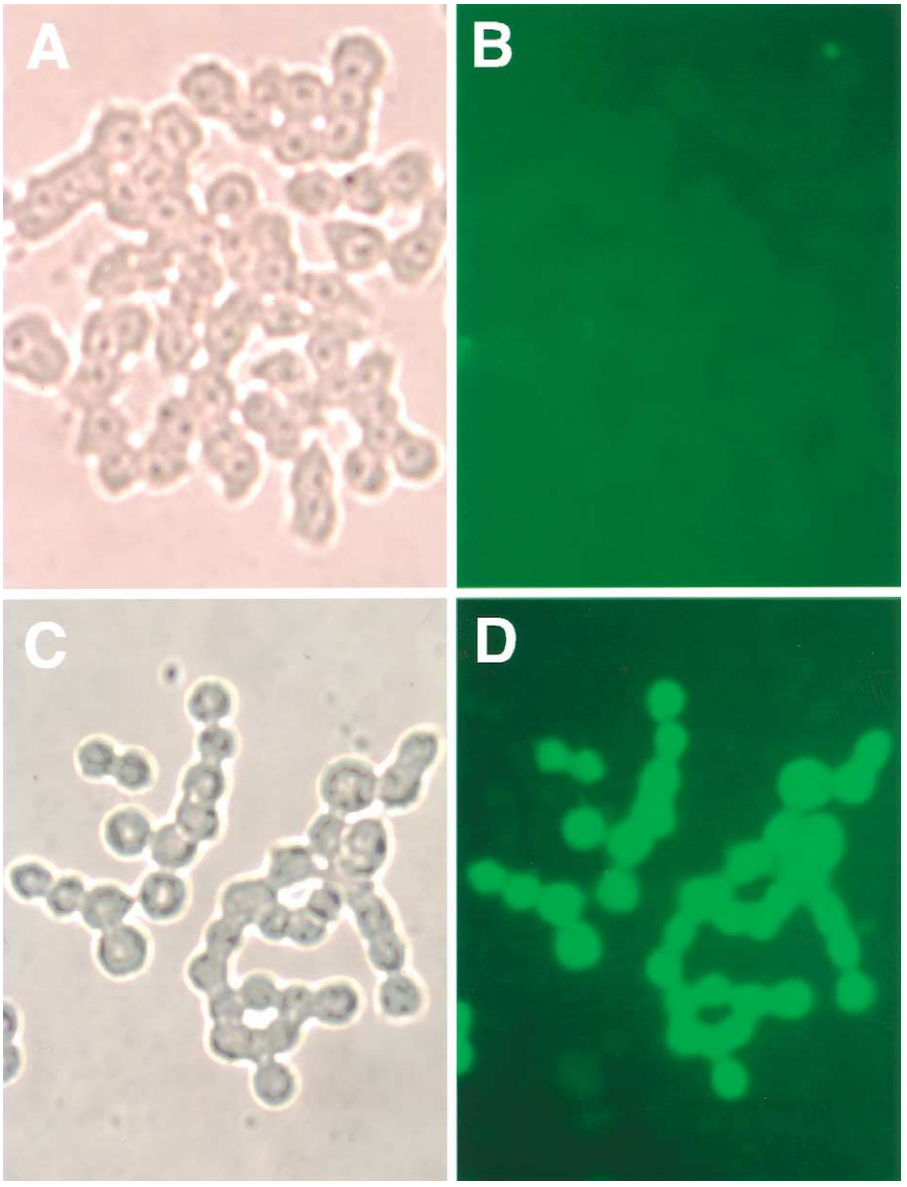


Fig. 6. Fluorescence microscopy of DOX accumulation and distribution assay. ME-V (A,B) and ME-2 (C,D) cells were loaded with DOX for 2 h (A,C, bright field; B,D, fluorescent field). Experiments were performed three times giving similar results. ×150.

rially diluted into ten concentrations. Every group of four wells received one drug concentration. After a 6-day incubation period, the cells were treated with 3-[4,5-dimethylthiazol-*Z*-yl]-2-5-diphenyl-tetrazolium bromide (MTT) which stains living cells. $IC_{50}$ was quantitatively evaluated as described [27].

### 2.9. Flow cytometry

Approximately $5\times10^5$/well of *WTH3* or the empty vector transfected cells were seeded in six-well dishes. Since the intracellular fluorescence intensity was tested at seven time points, seven wells for each transfectant group were prepared. One well for each group was not treated by DOX and served as the 0 time control. Each group of six wells was incubated with 30 μg/ml DOX in 1 ml of culture media for 2 h at 37°C in 5% $CO_2$. To measure the intracellular fluorescence intensity, the cells were washed twice in ice cold PBS and trypsinized. Cells were centrifuged at 1000 rpm for 5 min to pellet and then resuspended in 300 μl of 1% paraformaldehyde.

The intracellular DOX fluorescence content was analyzed by flow cytometry as described [33]. The fluorescence intensity was measured at 1- and 2-h intervals during the DOX incubation period. The remaining four wells of each transfectant were then washed twice with ice-cold PBS and incubated in a DOX-free culture medium for 4 h. The intensity of fluorescence at 1, 2, 3, and 4 h of chase was measured.

### 2.10. Fluorescence microscopic assay

Approximately $5\times10^4$/well of *WTH3* or the empty vector transfectants were cultured in six-well dishes. Individual wells were incubated with 10 μg/ml DOX in medium for 2 h. Medium was then removed and the cells were washed with ice-cold PBS, followed by the addition of fresh media without DOX. Cells were immediately examined for fluorescence at 488 nm excitation wavelength by fluorescence microscopy.

The GenBank accession number for *WTH3* is AF309646 (it will be released when the gene is published), and that for *W3* is AF11936.

## 3. Results

### 3.1. Isolation of a hypermethylated DNA fragments by MS-RDA

After two rounds of DNA subtractive hybridization and PCR amplification, individual fragments, considered difference products, were isolated from the MCF7/WT cells (Fig. 1A). These fragments were then subcloned into the pUC118 vector, and the candidate inserts were screened by amplicon Southern analysis. One of the candidate probes was *W3*, since it only hybridized with the tester, but not driver amplicon DNA (Fig. 1B). To prove that *W3* was hypermethylated in MCF7/AdrR cells, it was used as the probe to perform methylation sensitive Southern blot. Both cell lines' genomic DNAs were digested by *Hpa*II or the *Msp*I enzyme. *Msp*I is an isoschizomer of *Hpa*II, but not sensitive to $C^mCGG$. Therefore, *Msp*I could cleave a methylated site which could not be cut by *Hpa*II. Southern analysis results indicated that *W3* was hypermethylated in the MCF7/AdrR DNA because *Msp*I digestion released a band which was not cleaved by the *Hpa*II enzyme (Fig. 1B).

The *W3* fragment was sequenced and found to contain 573 bp which shared 89% and 96% identity with the corresponding portion of the human *Rab6* and *Rab6c*/*Rab6A′* genes [27–29]. Based on protein sequence analysis, *W3* encodes 175 amino acids without an interruption. In addition, at its 5′ end there is a stop codon, TAG, that is 27 bp away from a start codon. This suggested that the complete N terminus of the putative gene was included in the *W3* fragment, however, the C-terminal region was missing (Fig. 2A,B).

### 3.2. Obtaining the WTH3 gene by 3′-RACE

To acquire the putative full length cDNA of *W3*, the RACE technique was employed where a combined cDNA panel of different cell types (a commercial product of Clontech) was used as a template. To obtain the missing 3′ end portion of the candidate gene, a primer, PW3-1, paired with an antisense primer, PV-1, which was the vector's sequence close to the junction of the cDNA insertion, were utilized to perform PCR amplification. PW3-1 is a specific primer for *W3*, since in its 20 bp length sequence there are eight and three substitutions as compared to the corresponding region in *Rab6* and *Rab6c*. This specific feature was confirmed by the fact that no PCR product was generated from the plasmid containing *Rab6* or *Rab6c* sequences when PW3-1 and an antisense primer, a common sequence in *Rab6*, *Rab6c*, and *W3*, were used (data not shown). As a result of PCR amplification, a single 1.3 kb length PCR fragment was generated. This fragment was then electrophoresis-purified and sequenced. Our findings suggested that the PCR product was our target since the initial 349 bp sequence matched perfectly to the corresponding region of the *W3* sequence. Furthermore, in the middle of the PCR product, there was a stop codon which ended a 220-amino-acid stretch. To verify these results, another primer, PW3-RACE1, which is beyond *W3*'s 3′ region and before the stop codon, was used to perform a second RACE. PW3-RACE1 contains 4 bp substitutions as compared to the corresponding regions in *Rab6* and *Rab6c*, and is specific for the extended *W3* sequence. A PCR product approxi-

mately 800 bp in length was amplified. Sequence analysis confirmed that the N-terminal was identical to the corresponding sequence of the first PCR product. To further prove the accuracy of the PCR products, we generated the missing 3′ end portion of the target gene, *W3-3P*, from two other human cDNA libraries (Kidney and Brain cDNA libraries, Clontech). This was accomplished by utilizing the PW3-1 primer paired with the PW3-RACE2 primer which was located beyond the stop codon. Sequence analysis confirmed that both PCR products were identical to the two sequences previously generated by RACE. Therefore, we were convinced that the entire sequence information for the target gene had been obtained. We named this gene *WTH3* due to the fact that its truncated portion was originally isolated from MCF7/*WT* by *H*pa II cleavage MS-RDA.

To acquire an intact *WTH3* gene, the *W3-3P* fragment was digested with *Bgl*II and *Hin*dIII. The *Bgl*II site is an endogenous sequence, while the *Hin*dIII site was added into the PW3-RACE2 primer for cloning purposes. At the same time, the pUC118-W3 construction, which contains the *W3* DNA fragment isolated by MS-RDA, was also digested with *Bgl*II and *Hin*dIII to release the truncated region and create pUC118-W3$^{-}$. The enzymatic treated *W3-3P* was then ligated to the linearized pUC118-W3$^{-}$ to obtain the full-length *WTH3* gene. Differing from *Rab6* and *Rab6c*, which both encode a 208-amino-acid product, the *WTH3* cDNA encodes a 254-amino-acid protein. In the corresponding 718 bp sequences, *WTH3* is 92% and 95% identical to *Rab6* and *Rab6c* (Fig. 2A), while in the corresponding 208-amino-acid sequences, WTH3 is 89% and 91% identical to Rab6 and Rab6c (Fig. 2B). In addition, compared to the *Rab6* and *Rab6c* genes, *WTH3* has an elongated C-terminal region consisting of 46 amino acids (Fig. 2B). Recently, the Wiemann group reported that they have obtained 500 new cDNAs, among these, a *Rab6-like* gene was 100% identical to *WTH3* [30]. This finding verifies the sequence accuracy of the *WTH3* gene.

### 3.3. The WTH3 gene was underexpressed in MCF7/AdrR and MES-SA/Dx5 cells

Since the N-terminal portion of the *WTH3* gene, *W3*, was hypermethylated in MCF7/AdrR cells, the *WTH3* gene's expression level in MCF7/AdrR and MCF7/WT cells was analyzed by SQRT–PCR using gene-specific primers. The predicted size of the *WTH3* PCR product was 341 bp, while 284 bp was the anticipated length of the *G3PDH* gene which served as a quantitative control. The results showed that the expression of *WTH3* in MCF7/AdrR cells was 15 times less than that in MCF7/WT cells (Fig. 3). In addition, the *WTH3* gene's expression level was evaluated in the MDR cell line MES-SA/Dx5 and its non-MDR counterpart MES-SA. We found that *WTH3* was four times less expressed in MES-SA/Dx5 as compared to MES-SA cells (Fig. 3).

### 3.4. Drug-induced WTH3 transfectants growth inhibition assays

Hypermethylation and low expression of the *WTH3* gene in MCF7/AdrR and MES-SA/Dx5 cells indicated that this gene might be a negative regulator for drug resistance. To test this hypothesis and obtain reliable results, both MCF7/AdrR and MES-SA/Dx5 were transfected with the *WTH3* gene to generate stable cell lines. Another reason for using the MES-SA/Dx5 line was that it exhibited a much weaker MDR phenotype than MCF7/AdrR. Therefore, the *WTH3* gene could have a stronger influence on MES-SA/Dx5 than MCF7/AdrR cells.

The *WTH3* gene was generated by PCR and subcloned into the pcDNA3.1 vector to create pcDNA3.1/WTH3. This construction and the vector were separately introduced into the host cells by calcium phosphate precipitation procedure. The transfected cells were maintained in medium containing G418 for selecting stable transformed populations. After verifying, by measuring their $IC_{50}$s, that the stable populations harboring the transgene exhibited higher sensitivity to DOX than the controls integrated with the empty vector, limiting dilution was carried out to obtain stable cell clones. Three MCF7/AdrR and five MES-SA/Dx5 individual transfectants were selected. Limiting dilution procedures were also carried out to obtain five individual MCF7/AdrR or MES-SA/Dx5 transfectants integrated with the empty vectors (negative controls). The expression of the exogenous *WTH3* gene in each clone was con-

firmed by RT–PCR, where the gene-specific primer PW3-1 and pcDNA3.1 poly-linker primer PV-2 were used. All clones, along with their controls, were utilized for drug-induced cell growth inhibition assays. $IC_{50}$s for the anticancer drugs DOX, CISP, TAX, VBL, VCR, and VP-16 were evaluated. We found that the transgene increased the MCF7/AdrR clones' sensitivity to DOX, TAX, VBL, and VCR by factors ranging from 2- to 6-fold (data not shown). However, the transgene had a much stronger influence on four out of five MES-SA/Dx5 clones. For example, both clones #2 (ME-2) and #8 (ME-8) had significant increased sensitivity to DOX, TAX, VBL, VCR, and VP-16 as compared to the five controls containing the empty vector (ME-V). The sensitivity of clones #4 (ME-4) and #7 (ME-7) to the same drugs was also elevated by the transgene but to a lesser extent. Clone #5, which expressed a very low level of the transgene, exhibited a drug resistant phenotype similar to that of all five control sublines (data not shown). In the next paragraph, detailed information on the $IC_{50}$ measurements of clones ME-2, ME-4, and ME-7 is presented.

To determine the $IC_{50}$s, ME-2, ME-4, ME-7, and five ME-V sublines were kept in media without the drug, or with the other ten drug concentrations for 6 days. More than three individual experiments were performed for each cloned cell line. The $IC_{50}$ measurements found that the *WTH3* transgene increased the host clone's sensitivity to DOX, TAX, VBL, VCR, and VP-16, but not to CISP, to varying degrees as compared to the control cell lines which exhibited the original MDR phenotype (Table 1). Since all five ME-V controls had similar resistance to the drugs tested, only the results obtained from one ME-V were listed. The sensitivity of the ME-2 clone to DOX, TAX, VBL, VCR, and VP-16 was 35, 216, 45, 174, and 26 times greater than that of the control cells (Table 1 and Fig. 4A). The ME-4 clone's sensitivity to the same anticancer drugs was 8, 22, 8, 13, and 4 times higher than the control cells, while that of the ME-7 clone was elevated 14, 30, 16, 28, and 8.5 times relative to the control (Table 1). Expression of the *WTH3* transgene in ME-2, ME-4, and ME-7 was verified by RT–PCR (Fig. 4B). Densitometer analysis found that the expression level of the transgene in ME-2 was 1.6 and 2.2 times higher than that in ME-7 and ME-4, which could be a reason for ME-2 exhibiting higher drug sensitivity.

### 3.5. Flow cytometry assay for DOX uptake and retention in ME-2 cells

DOX is fluorescent, and this attribute provides easy monitoring of its intracellular accumulation by flow cytometry. Thus, ME-V and ME-2 cells were incubated with DOX for 2 h, after which the cells were washed and remained in a medium without DOX for 4 h. DOX uptake and retention in the cells was quantitatively determined at different time points (see Section 2). The ME-V cells displayed no significant increase in cellular fluorescence after DOX incubation. The ME-2 cells, which contained the *WTH3* transgene, however, displayed greatly increased DOX uptake (Fig. 5A). Furthermore, the intensity of fluorescence in ME-2 and ME-V cells was measured at four time points after the cells were washed with PBS buffer. The results showed that the fluorescence remained much stronger even after 4 h of chase in ME-2 than that in ME-V cells (Fig. 5B). These findings clearly demonstrate the positive effect of the *WTH3* gene on DOX uptake and retention in the host cells.

### 3.6. Fluorescence microscopy of DOX accumulation in ME-2 cells

Flow cytometry experiments found that the *WTH3* gene stimulated the host cells' uptake and retention of DOX. To visually determine the intercellular location of the accumulated DOX, fluorescence microscopy was performed. The ME-V and ME-2 cells were treated with DOX for 2 h, after which the drug was washed away. The fluorescence accumulation and distribution in the control and test cells were examined under a microscope (Fig. 6A–D). It was discovered that the control cells with the drug resistant phenotype only contained trace amounts of fluorescence (Fig. 6B), while strong fluorescence was displayed in the nucleus and cytoplasm of the ME-2 cells (Fig. 6D). The intensity of fluorescence continued to remain strong even after 3 h of chase in the ME-2 cells (data not shown).

## 4. Discussion

The *WTH3* gene was discovered due to hypermethylation of its N-terminal portion in MCF7/AdrR cells. Function analysis suggests that this gene could be involved in cellular MDR phenotype development.

*WTH3* is homologous to the human genes, *Rab6* and *Rab6c*. *Rab6* is thought to control intra-Golgi transport by acting as an inhibitor [34]. Our previous studies suggest that *Rab6c* is involved in developing the MDR phenotype in MCF7/AdrR cells [27]. *Rab6c* was also named *Rab6A′* in a recent publication where it has been suggested that it is an isoform of *Rab6* due to alternative splicing. However, *Rab6A′* is not able to stimulate Golgi-to-endoplasmic reticulum retrograde transport as described previously for *Rab6* [29]. Both *Rab6* and *Rab6c* encode 208 amino acids with only three substitutions between them. *WTH3* encodes 254 amino acids with 22 and 19 substitutions as compared to Rab6 and Rab6c. These substitutions are evenly distributed, and this would indicate that *WTH3* is not an alternative spliced product of *Rab6*. Four domains of the K-ras protein involved in GTP/GDP binding are also included in WTH3 (corresponding to residues 20–27, 67–73, 123–130, and 153–159) [28]. This implies that *WTH3* encodes a small G protein. However, differing from Rab6 and Rab6c, WTH3 does not possess any cysteine near its COOH terminus. The existence of cysteine is assumed to be necessary for many G proteins' fatty acylation, membrane association, and biological function [35]. The C-terminal diversification suggests that the *WTH3* protein might have its own unique utilities, even though *WTH3* and *Rab6c* do share some biological functions.

Our studies demonstrated that the N-terminal portion of *WTH3* was hypermethylated in MCF7/AdrR and the gene was less expressed in MCF7/AdrR and MES-SA/Dx5 cells than in their parental cell lines. In addition, based on information provided by the Wiemann group, the 398 bp length region located at the N-terminus of *WTH3* was not only GC-rich ($\sim$71% of GC content) but also contained CpG islands [30]. Although the promoter of *WTH3* has not yet become available, its expression could be regulated by DNA methylation. This is because hypermethylation in promoters is often accompanied by hypermethylation in the 5′ transcribed region, and CpG islands usually extend from promoters into the 5′ transcribed region [20,36–38]. It is expected that when the promoter of *WTH3* is found, a reverse correlation between DNA methylation and gene expression will be ascertained.

To test whether increasing the *WTH3* gene transcript in MCF7/AdrR and MES-SA/Dx5 cells could convert the MDR phenotype, stable cloned cell lines were established. Several clones from each cell line were generated and used to perform drug-induced cell death experiments. The results demonstrated that the gene influenced both MDR cell lines although the effect is greater on MES-SA/Dx5 than MCF-7/AdrR. The mechanism for this disparity remains unclear. In the past, we reported that the *Rab6c* gene was involved in MDR in MCF7/AdrR cells. To date, our results suggest that another *Rab6*-like gene, *WTH3*, was also related to the MDR phenotype in two human cell lines. Differing from known MDR genes, which function as positive regulators for MDR development, the *Rab6* genes play negative regulation roles. It will be interesting to understand the biological pathway(s) of each member in the *Rab6* gene family and explore the possible relationships between them and P-glycoprotein. It has been reported that the mammalian *Rab6* gene product was located in the medial and *trans* Golgi cisternae as well as the *trans* Golgi network [29,34,39], while P-glycoprotein was localized in the plasma membrane of drug-resistant cells. However, whether there is direct or indirect interaction between Rab6 and P-glycoprotein remains unclear.

The question of whether the WTH3 protein directly interacts with P-glycoprotein also remains to be determined. This is because, differing from Rab6, WTH3 does not possess any cysteine near its carboxyl-terminal region, suggesting it might have a different cellular localization. The cellular location of WTH3 could conceivably be determined by tagging it with certain markers, such as green fluorescence protein (GFP), or, most ideally, by an antibody which could only recognize WTH3, but not Rab6 and Rab6c. When such an antibody is available, it will assist the study of determining the possible relationship between WTH3 and P-glycoprotein.

Based on our data, it is necessary to further investigate the *Rab6* genes' (*Rab6*, *Rab6c*, and *WTH3*)

involvement in MDR evolution. The results of this study could have a great impact in understanding clinical MDR development.

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## References

[1] C.J. Chen, J.E. Chin, K. Ueda, D.P. Clark, I. Pastan, M.M. Gottesman, I.B. Roninson, Cell 47 (1986) 381–389.
[2] P. Gros, Y.B.B. Neriah, J.M. Croop, D.E. Housman, Nature 323 (1986) 728–731.
[3] P. Gros, J. Croop, D. Housman, Cell 47 (1986) 371–380.
[4] S.P. Cole, G. Hardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M. Duncan, R.G. Deeley, Science 258 (1992) 1650–1654.
[5] G.J. Zaman, M.J. Flens, M.R. van Leusden, M. de Haas, H.S. Mulder, J. Lankelma, H.M. Pinedo, R.J. Scheper, F. Baas, H.J. Broxterman, P. Borst, Proc. Natl. Acid. Sci. USA 91 (1994) 8822–8826.
[6] H. Blohuis, H.W. van Veen, B. Poolman, A.J. Driessen, W.N. Konings, FEMS Microbiol. Rev. 21 (1997) 55–84.
[7] M.A. Izquierdo, A.G.J. van der Zee, J.B. Vermorken, P. ven der Valk, J.A.M. Belien, G. Giaccone, G.L. Scheffer, M.J. Flens, H.M. Pinedo, P. Kenemans, C.J.L.M. Meijer, E.G.E. deVries, R.J. Scheper, J. Natl. Cancer Inst. 87 (1995) 1230–1235.
[8] M. Izquierdo, G. Scheffer, M. Flens, G. Giaccone, H.J. Broxterman, C.J.L.M. Meijer, P. van der Valk, R.J. Scheper, Am. J. Pathol. 148 (1986) 877–887.
[9] K.D. Tew, Cancer Res. 54 (1994) 4313–4320.
[10] K. Nooter, G. Stoter, Pathol. Res. Pract. 192 (1996) 768–780.
[11] S.B. Kaye, Curr. Opin. Oncol. 10 (1998) S15–19.
[12] A.D. Riggs, P.A. Jones, Adv. Cancer Res. 40 (1983) 1–30.
[13] C.H. Spruck, W.M. Rideout, P.A. Jones, in: J.P. Jost, H.P. Saluz (Eds.), DNA Methylation, Birkhauser, Basel, 1993, pp. 487–509.
[14] S.B. Baylin, J.G. Herman, J.R. Graff, P.M. Vertino, J.P. Issa, Adv. Cancer Res. 72 (1998) 141–196.
[15] J. Nyce, Cancer Res. 49 (1989) 5829–5836.
[16] J. Nyce, S. Leonard, D. Canupp, S. Schulz, S. Wong, Proc. Natl. Acad. Sci. USA 90 (1993) 2960–2964.
[17] L. Desiderato, M.W. Davey, A.A. Piper, Somat. Cell Mol. Genet. 23 (1997) 391–400.
[18] H. Kusaba, M. Nakayama, T. Harada, K. Torigoe, E.D. Green, S.W. Scherer, K. Kohno, M. Kuwana, M. Wada, Somat. Cell Mol. Genet. 23 (1997) 259–274.
[19] G. Strathdee, M.J. MacKean, M.M. Illand, R. Brown, Oncogene 18 (1999) 2335–2341.
[20] A.P. Bird, Nature 321 (1986) 209–213.
[21] F. Larsen, G. Gunderson, R. Lopez, H. Prydz, Genomics 13 (1992) 1095–1107.
[22] F. Antequera, A.P. Bird, Proc. Natl. Acad. Sci. USA 90 (1993) 11995–11999.
[23] L. Yuan, J. Shan, D. De Risi, J. Broome, J. Lovecchio, D. Gal, V. Vinciguerra, H.P. Xu, Cancer Res. 59 (1999) 3215–3221.
[24] T. Ushijima, K. Morimura, Y. Hosoya, H. Okonogi, M. Tatematsu, T. Sugimura, M. Nagao, Proc. Natl. Acad. Sci. USA 94 (1997) 2284–2289.
[25] N. Lisitsyn, N. Lisitsyn, M. Wigler, Science 259 (1993) 946–951.
[26] G. Batist, A. Tulpule, B.K. Sinha, A.G. Katki, C.E. Myers, K.H. Cowan, J. Biol. Chem. 261 (1986) 15544–15549.
[27] J. Shan, J.M. Mason, L. Yuan, M. Barcia, D. Porti, A. Calabro, D. Budman, V. Vinciguerra, H.P. Xu, Gene 257 (2000) 67–75.
[28] A. Zahraou, N. Touchot, P. Chardin, A. Tavitian, J. Biol. Chem. 264 (1989) 12394–12401.
[29] A. Echard, F.J. Opdam, H.J. de Leeuw, F. Jollivet, P. Savelkoul, W. Hendriks, J. Voorberg, B. Goud, J.A. Fransen, Mol. Biol. Cell 11 (2000) 3819–3833.
[30] S. Wiemann et al., Genome Res. 11 (2001) 422–435.
[31] W.G. Harker, F.R. MacKintosh, B.I. Sikic, Cancer Res. 43 (1983) 4943–4950.
[32] W.G. Harker, B.I. Sikic, Cancer Res. 45 (1985) 4091–4096.
[33] L.A. Doyle, D.D. Ross, R. Sridhara, A.T. Fojo, S.H. Kaufmann, E.J. Lee, C.A. Schiffer, Br. J. Cancer 71 (1995) 52–58.
[34] O. Martinez, C. Antony, G. Pehau-Arnaudet, E.C. Berger, J. Salamero, B. Goud, Proc. Natl. Acad. Sci. USA 94 (1997) 1828–1833.
[35] M. Barbacid, Annu. Rev. Biochem. 56 (1987) 779–827.
[36] A. Razin, H. Cedar, Cell 77 (1994) 473–476.
[37] Z. Siegfried, H. Cedar, Curr. Biol. 7 (1997) R305–307.
[38] S.U. Kass, D. Pruss, A.P. Wolffe, Trends Genet. 13 (1997) 444–449.
[39] J. Van Wye, N. Ghori, P. Webster, R.R. Mitschler, H.G. Elmendorf, K. Haldar, Mol. Biochem. Parasitol. 83 (1996) 107–120.